REMARKS

I. Pending Claims

Claims 5, 7-9, 12, 15 and 16 are canceled as directed to a non-elected invention.

Claims 1-4, 6, 10, 11, 13 and 14 are pending.

II. Amendments

A statement describing related patent applications has been added.

Claims 1, 2 and 3 have been rewritten to clarify the claimed invention with more particularity, more specifically identifying the subject matter of the claim. Applicant's assert that it is not reasonable or practical to explicitly state or otherwise claim any and all substantial equivalents of the claimed invention (*Festo v. Shoketsu Kogyo Kabushiki Co. Ltd. et al.* 535 US ___ 2002).

Claim 1 has been amended to claim an agent comprising a ligand polypeptide which has a particular amino acid sequence selected from:

(1) the amino acid sequence represented by SEQ ID NO:73.

Support for this is found in the specification and claims as originally filed.

(2) an amino acid sequence wherein 1 to 15 contiguous amino acid residues are deleted from the amino acid sequence represented by SEQ ID NO: 73.

Support for this is found in the specification as filed, for example on page 21, lines 29-31.

(3) an amino acid sequence wherein 1 to 80 contiguous amino acid residues are added to the amino acid sequence represented by SEQ ID NO: 73.

Support for this is found in the specification as filed, for example on page 21, lines 32-34.

(4) an amino acid sequence wherein 1 to 15 amino acid residues are conservatively substituted with other amino acid residues in the amino acid sequence represented by SEQ ID NO: 73.

Support for this is found in the specification as filed, for example on page 21, line 35 to page 22 line 2, or page 19, line 31 to page 20, line 9.

Claim 2 has been amended to claim an agent comprising a ligand polypeptide which has a particular amino acid sequence selected from:

(1) the amino acid sequence of SEQ ID NO:74.

Support for this is found in the specification and claims as filed, for example on page 30, lines 13-15.

(2) the amino acid sequence represented by SEQ ID NO:74 fused to the N-terminal of SEQ ID NO:73.

Support for this is found in the specification and claims as filed, for example on page 22, lines 7-12.

(3) the amino acid residues 2 to 21 of SEQ ID NO:73.

Support for this is found in the specification as filed, for example on page 29, lines 20-22.

(4) the amino acid residues 3 to 21 of SEQ ID NO:73.

Support for this is found in the specification as filed, for example on page 29, lines 23-24.

(5) the amino acid residues 4 to 21 of SEQ ID NO:73.

Support for this is found in the specification as filed, for example on page 29, lines 24-26.

(6) the amino acid residues 5 to 21 of SEQ ID NO:73.

Support for this is found in the specification as filed, for example on page 29, lines 26-28.

(7) the amino acid residues 6 to 21 of SEQ ID NO:73.

Support for this is found in the specification as filed, for example on page 29, lines 28-30.

(8) the amino acid residues 7 to 21 of SEQ ID NO:73.

Support for this is found in the specification as filed, for example on page 29, lines 30-32.

(9) the amino acid residues 8 to 21 of SEQ ID NO:73.

Support for this is found in the specification as filed, for example on page 29, lines 32-34.

(10) the amino acid residues 9 to 21 of SEQ ID NO:73.

Support for this is found in the specification as filed, for example on page 29, lines 34-36.

(11) the amino acid residues 10 to 21 of SEQ ID NO:73.

Support for this is found in the specification as filed, for example on page 30, lines 1-2.

(12) the amino acid residues 11 to 21 of SEQ ID NO:73.

Support for this is found in the specification as filed, for example on page 30, lines 2-4.

(13) the amino acid residues 12 to 21 of SEQ ID NO:73.

Support for this is found in the specification as filed, for example on page 30, lines 4-6.

(14) the amino acid residues 13 to 21 of SEQ ID NO:73.

Support for this is found in the specification as filed, for example on page 30, lines 6-8.

(15) the amino acid residues 14 to 21 of SEQ ID NO:73.

Support for this is found in the specification as filed, for example on page 30, lines 8-10.

(16) the amino acid residues 15 to 21 of SEQ ID NO:73.

Support for this is found in the specification as filed, for example on page 30, lines 10-12.

Claim 3 has been amended to claim an agent comprising a ligand polypeptide which has a particular amino acid sequence selected from SEQ ID NO: 5, SEQ ID NO: 8, SEQ ID NO: 47, SEQ ID NO: 50, SEQ ID NO: 61 or SEQ ID NO: 64.

Support for this is found in the specification and claims as filed, as for example on page 20, lines 20-25 or page 30, lines 16-22.

Claims 4, 6, 10, 11 and 13 have been amended to phrase the claims as a method, in accordance with accepted US Patent practice.

Claim 14 has been amended to more clearly recite the claimed method, in accordance with accepted US Patent practice.

Newly added claims 17-19 encompass subject matter of the claims as originally filed, as previously claimed by amended claim 1 and dependent claims thereof prior to the requested amendments.

No new matter is added by these amendments.

No change of inventorship is required by these amendments.

II. Traverse

A) Objections to the Specification

The objection to the specification has been addressed and overcome by the amendment above requesting insertion of the appropriate reference to related applications.

The objection to the Abstract as been addressed and overcome by the herewith submitted substitute page.

B) Rejection under §101

The rejection of claim 13 has been overcome by amendment above to rewrite the claim as a method.

C) Rejection under §112 1st paragraph

Applicants traverse the rejection in view of the amended claims. The claims as amended clearly recite the specific amino acid sequences that would be encompassed by the claimed invention such that one of ordinary skill in the art would be able to make and practice the claimed invention. The Examiner's personal opinions are not sufficient support for the asserted rejection.

Contrary to the Examiner's assertion (page 6, para. 1), the specification clearly provides the information for the structure and asserted function of the claimed invention. This information is found throughout the specification, and in particular on pages 20-22 or 29-30 for example. One of ordinary skill in the art, at the time of the invention, could understand and practice the claimed invention from the disclosure provided.

Given the high level of skill of one of ordinary skill in the art at the time of the invention, only routine testing would be required to ascertain efficacy or functionality. Methods for making, isolating and testing the claimed invention are described in the specification such that one of ordinary skill in the art would only require routine testing to ascertain the invention.

The claims as amended are defined and limited in scope. The level of skill in the art of protein expression/production at the time of the invention is sufficiently high that the specific variants of the claimed invention are recited with sufficient particularity that one of ordinary skill in the art could ascertain and create each and every species of the claimed invention without undue experimentation.

Given the demonstrated structure, function and activity of the exemplar species of protein of the claimed invention described in detail in the specification, one of ordinary skill in the art would find sufficient guidance, and direction to prepare the species of the claimed invention.

The rejection ignores the full scope of the teaching of the disclosure by mischaracterizing the level of skill of one of ordinary skill in the art at the time of the invention. The rejection is unsupported by any evidence that contradicts the teaching of the disclosure. By applying standards that are unsupported by evidence, the rejection would be based upon outdated standards not reflective of the state of the art at the time of the invention as described in the specification and known in the art.

Applicants submit for the Examiner's review two peer-review scientific journal articles which describe the experiments of the disclosure and which support the applicants assertion.

Hinuma, S. et al., "A prolactin-releasing peptide in the brain" *Nature* 393:272-276 (May 21, 1998) describes the production and testing of peptides of the invention. Figure 2 of the article identifies peptides which correspond to the claimed invention that were tested.

Kawamata, Y. et al., "Analyses for Susceptibility of Rat Anterior Pituitary Cells to Prolactin-Releasing Peptide" *Endocrine* 12:215-221 (June 2000) describes further experiments that demonstrate activity of the peptides of the claimed invention (see for example Fig. 5 therein).

The rejection should be withdrawn.

D) Rejection under §112 2nd paragraph

Applicants believe the claims as amended have overcome these rejections.

Applicants have amended the term "modulating" to recite "promote" without prejudice to filing future continuing applications.

Applicants have amended the term "represented" to recite "of".

Applicants have amended Claim 2 to recite the specific species in place of "substantial equivalent" without prejudice.

Claim 6 has been amended to conform to the elected species.

Claims 11 and 14 have been amended to recite to method steps.

E) Rejection under §103(a)

Claim 1 has been amended to particularly recite the limitation of the amino acid sequence of SEQ ID NO:73.

The asserted art combined as suggested by the Examiner do not teach or suggest the claimed invention. This rejection should be withdrawn.

IV. Conclusion

Reconsideration of the claims as amended in view of the traverse made above is solicited. Early allowance of the claims is requested. Should the Examiner believe that a conference with applicants' attorney would advance prosecution of this application, the Examiner is respectfully invited to call applicants' attorney.

Respectfully submitted,

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Analyses for Susceptibility of Rat Anterior Pituitary Cells to Prolactin-Releasing Peptide

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We validated the effect of prolactin-releasing peptide (PrRP) on prolactin (PRL) secretion from rat anterior pituitary cells in in vitro culture. We found that culture conditions considerably influenced the response of the anterior pituitary cells to PrRP. Longer culture term (4 d) was required to obtain better responses of the anterior pituitary cells to PrRP in comparison to thyrotropin-releasing hormone (TRH). Under the culture conditions employed here, PrRP was comparable to TRH in the potency promoting PRL secretion, and the action of PrRP was very specific for PRL secretion. The susceptibility of the anterior pituitary cells to PrRP varied in female rats depending on the process of reproduction: the cells prepared from lactating rats were the most sensitive to PrRP compared with those from random-cycle and pregnant rats. Because the expression levels of PrRP receptor mRNA in the pituitary varied during the reproductive process, we speculated that the susceptibility of the anterior pituitary cells would reflect cellular changes including the expression level of PrRP receptors. In addition, treatment with estrogen in vivo enhanced the susceptibility of the cultured anterior pituitary cells in male rats. Our results indicate that the susceptibility of the rat anterior pituitary cells to PrRP is regulated by physiological mechanisms.

Key Words: Prolactin-releasing peptide; seven-transmembrane-domain receptor; prolactin; pituitary.

Introduction

Prolactin (PRL) secretion from the pituitary gland is under the complex regulation of a broad array of factors *(1)*. Although a major mechanism for regulating PRL secretion is believed to be inhibitory control by dopamine, some studies have indicated that unidentified hypothalamic factors might play important roles in the stimulatory control of PRL secretion *(1–5)*. So far many neuropeptides, e.g., thyrotropin-releasing hormone (TRH), oxytocin, vasopressin, and neurotensin, reportedly could promote PRL release in vitro or in vivo *(1)*. TRH is a representative of such factors, but its main physiological role in the pituitary is undoubtedly to stimulate the secretion of thyroid-stimulating hormone (TSH). As in the case of TRH, none of the known factors shows a stimulating activity on PRL secretion in a specific manner.

We have recently established a strategy to identify endogenous ligands for orphan seven-transmembrane-domain receptors (7TMRs) and succeeded in identifying an endogenous ligand for the orphan 7TMR, hGR3 *(6,7)*. We originally isolated hGR3 from the human pituitary and subsequently have found that its rat counterpart, UHR-1 *(8)*, was most abundantly expressed in the anterior lobe of the pituitary when examined by reverse transcriptase-polymerase chain reaction (RT-PCR) and *in situ* hybridization, although UHR-1 mRNA was also detected in other tissues including the central nervous system *(9)*. Because the endogenous ligand of hGR3 showed specific PRL release–promoting activity to the rat anterior pituitary cells in in vitro culture, we named it PRL-releasing peptide (PrRP) *(7)*.

However, Samson et al. *(10)* have recently reported that the stimulatory activity of PrRP on PRL secretion was very weak in comparison with that of TRH in in vitro experiments using primary-cultured rat anterior pituitary cells prepared from both intact male and random-cycle female rats. They are hence dubious about the physiological significance of PrRP in the regulation of PRL secretion. In this article, we report on the culture conditions under which PrRP could show a potent and specific PRL release–promoting activity to the primary-cultured rat anterior pituitary cells. After determining proper culture conditions, we compared the effects of PrRP and TRH on PRL secretion. We show here that the susceptibility of the anterior pituitary cells was changed in female rats during the reproductive process or in male rats by treatment with estrogen. In addi-

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tion, we show that the expression level of PrRP receptor mRNA was changed in the pituitary in female rats during the reproductive process and discuss the possible involvement of PrRP in the physiological regulation of PRL secretion.

Results

We first determined adequate culture conditions under which the action of PrRP on PRL secretion from primary-cultured rat anterior pituitary cells was evidently detectable. We prepared rat anterior pituitary cells according to the method reported by Vale et al. *(11)* and Shiota et al. *(12,13)* with minor modifications. We used collagenase and pancreatin for the mild dispersion of the anterior pituitary, and cultured the obtained cells in poly-D-lysine-coated plates for the efficient attachment of the cells. We used principally lactating Fischer rats as a donor for the anterior pituitaries, because these provided reproducible results in the assessment of PRL release–promoting activity in PrRP. As shown in Fig. 1, PrRP at 10^{-7} *M* slightly enhanced PRL release (i.e., about 120% compared with the control) from the rat anterior pituitary cells in the culture for 1 d. On the other hand, the effect of PrRP on PRL secretion was more evident (i.e., about 230% compared with the control) in the culture for 4 d than in that for 1 d. By contrast, the effect of TRH on PRL secretion was almost comparable (about 160–170% compared with the control) between the 1-d and 4-d culture. These results indicate that the culture term influences differently the susceptibility of the rat anterior pituitary cells to PrRP and TRH, respectively. We obtained a good response of the rat anterior pituitary cells to PrRP even after the culture for 1 wk under the conditions employed here (data not shown). Based on these results, we decided to culture the cells for 4 d to elucidate the action of PrRP on PRL secretion.

Under the same culture conditions (i.e., culture term for 4 d), we examined the effect of PrRP on the release of other pituitary hormones. As shown in Fig. 2, PrRP did not influence the secretion of growth hormone (GH), TSH, adrenocorticotropic hormone (ACTH), luteinizing hormone (LH), or follicle-stimulating hormone (FSH), even though under the same conditions growth hormone–releasing hormone (GHRH), TRH, corticotropin-releasing hormone (CRH), and luteinizing hormone–releasing hormone (LHRH) evidently promoted the secretion of these hormones, respectively. These results indicate that PrRP behaves as a specific factor for promoting PRL secretion at least in in vitro culture.

We subsequently compared the kinetics of PRL release induced by PrRP and TRH. PrRP significantly promoted PRL release within 15 min. The maximum PRL release induced by PrRP was observed between 15 min and 1 h (Fig. 3A). We therefore decided to treat the cells for 20 min to 1 h when determining the PRL release–promoting activity of PrRP in the following experiments. The increase in

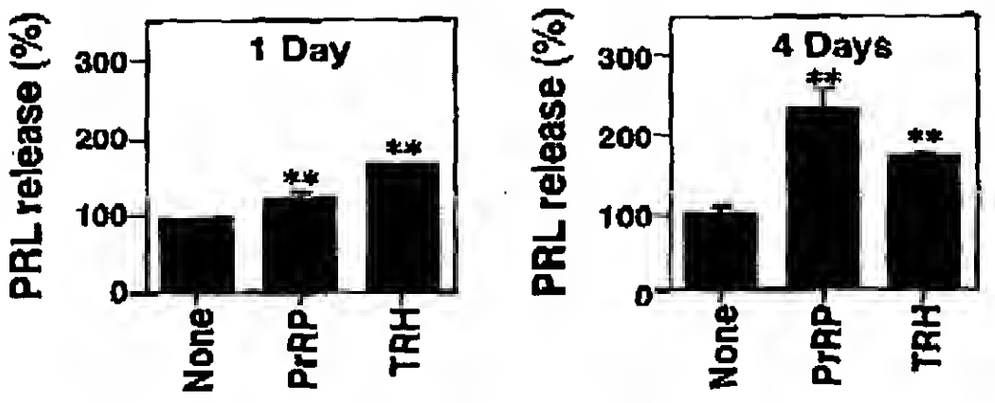

Fig. 1. Effects of culture term on PRL release from rat anterior pituitary cells by PrRP and TRH. Anterior pituitary cells prepared from lactating rats were cultured for 1 d **(left)** and 4 d **(right)**. These cells were treated with or without samples (rat PrRP31 or TRH at 10^{-7} *M*) for 1 h, and then PRL concentrations in the culture supernatants were determined. PRL concentrations in the control without the sample were 88.8 and 396 ng/mL after 1-d and 4-d culture, respectively. Data are expressed as mean values ± SEM of percentages in PRL release with each sample in comparison with the control in multiple assays ($n=4$), except for the control of 1-d culture ($n=3$). ** $p<0.01$; Student's *t*-test, when compared with the control.

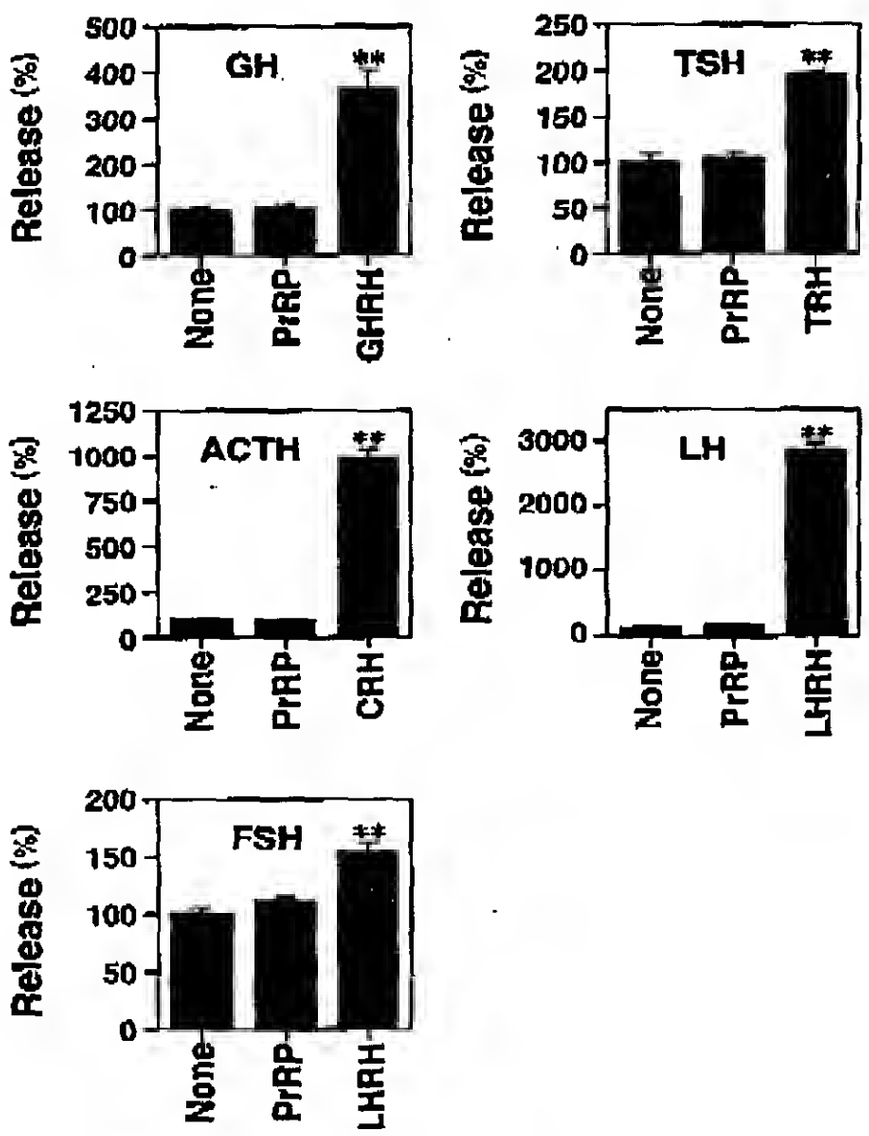

Fig. 2. Effects of PrRP on other anterior pituitary hormone secretions. Anterior pituitary cells were prepared from lactating rats and cultured for 4 d. Culture supernatants were then collected 3 h after adding the indicated peptide samples at 10^{-7} *M* except for GHRH at 10^{-9} *M*. Bovine and rat PrRP31 were used in the analyses for TSH and FSH secretions and GH, LH, and ACTH secretions, respectively. Data are expressed as mean values ± SEM of percentages in release with each sample in comparison with the control in multiple assays ($n=3$ to 4). ** $p < 0.01$; Student's *t*-test, when compared with the control.

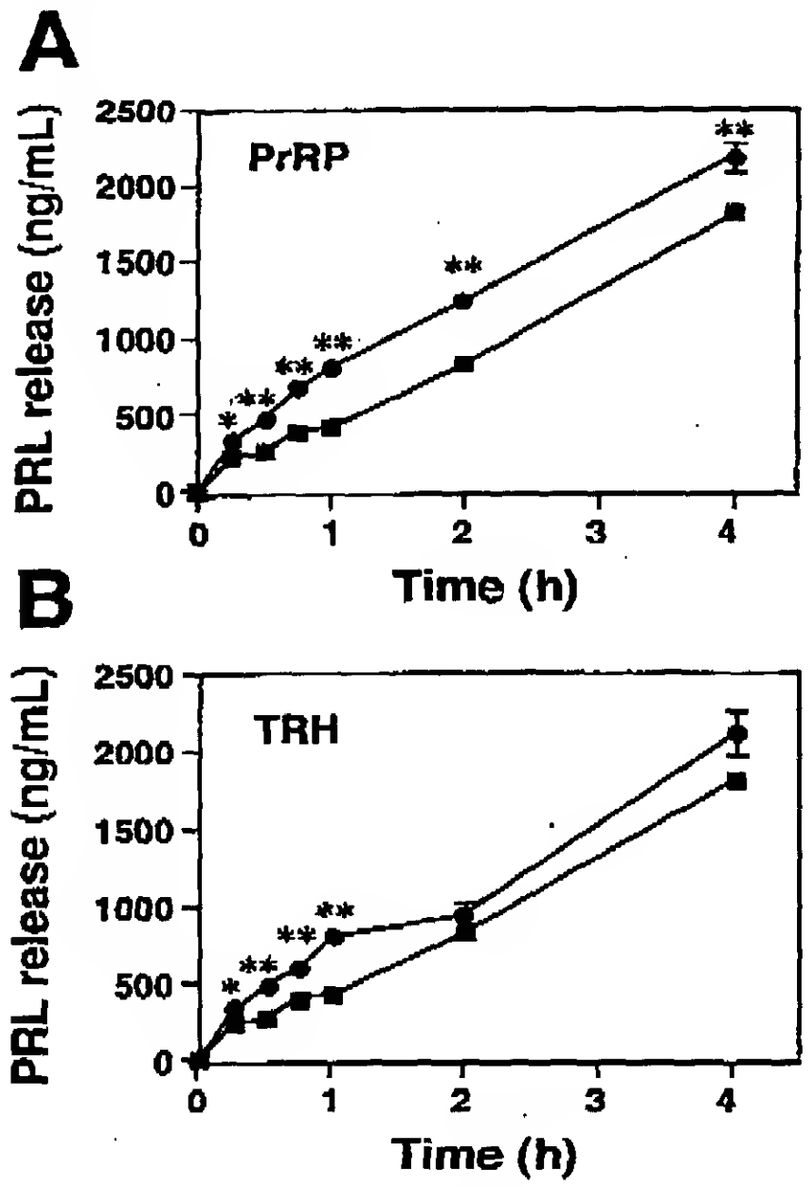

Fig. 3. Kinetics of PRL secretion from rat anterior pituitary cells after stimulation with PrRP (A) or TRH (B). Anterior pituitary cells prepared from lactating rats were cultured under the same conditions as in Fig. 2. After the culture without (■) or with (●: bovine PrRP31 or TRH) a sample at 10^{-6} *M*, supernatants were harvested at the time indicated, and then their PRL concentrations were determined. Data are expressed as mean values ± SEM nanograms/milliliter in PRL release with each sample in comparison with the control without samples in multiple assays ($n = 4$). *$p < 0.05$; **$p < 0.01$; Student's *t*-test, when compared with the control.

PRL release induced by TRH also reached the maximum around 1 h under the same experimental conditions (Fig. 3B). We examined whether or not there were differences in the effects of bovine, human, and rat PrRPs on PRL release from the rat anterior pituitary cells. PrRP31 derived from different species showed similar potency in the promotion of PRL secretion (Fig. 4). There were no apparent differences in the potency between PrRP31 and PrRP20 (Fig. 5).

To analyze whether or not the response of the anterior pituitary cells to PrRP would change during reproductive stages, we prepared the cells from random-cycle, pregnant, and lactating female rats, respectively, and examined their responses to PrRP. As shown in Fig. 6, PRL release promoted by PrRP at both 10^{-8} and 10^{-7} *M* was significantly greater in the anterior pituitary cells prepared from lactating rats than in those from random-cycle or pregnant female

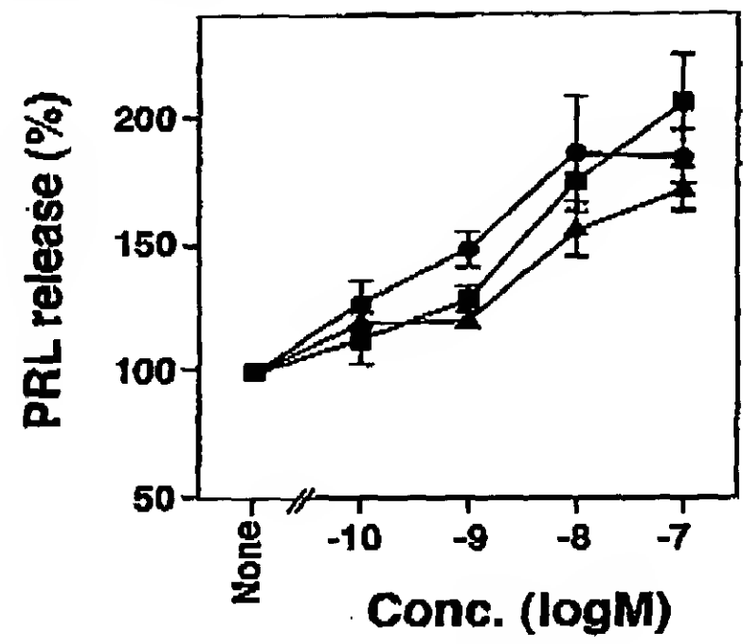

Fig. 4. Comparison of PRL secretion-stimulatory activities of bovine, human, and rat PrRPs. Anterior pituitary cells prepared from lactating rats were cultured under the same conditions as in Fig. 2. After the cells were treated with or without bovine (▲), human (●), and rat (■) PrRP31 at the indicated concentrations for 1 h, PRL concentrations in the culture supernatants were determined. Data are expressed as mean values ± SEM of percentages in PRL release with each sample in comparison with the control in multiple assays ($n = 4$).

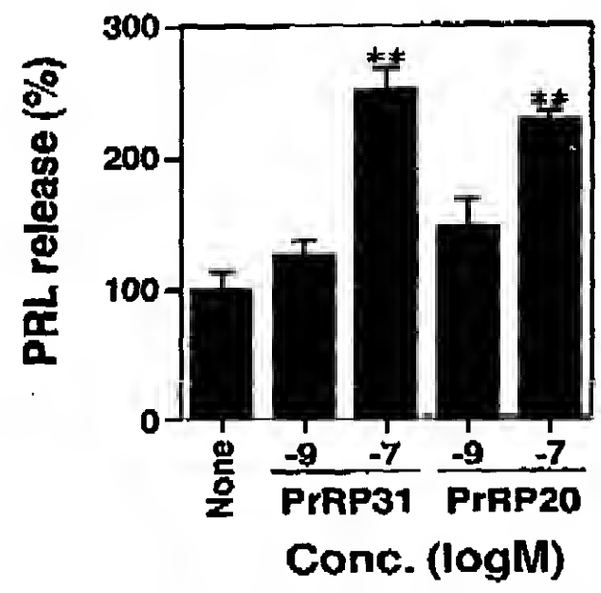

Fig. 5. Comparison of PRL secretion-stimulatory activities of PrRP31 and PrRP20. Anterior pituitary cells prepared from lactating rats were cultured under the same conditions as in Fig. 2. After the cells were treated with or without bovine PrRP31 and PrRP20 at the indicated concentrations for 20 min, PRL concentrations in the culture supernatants were determined. Data are expressed as mean values ± SEM of percentages in PRL release with each sample in comparison with the control in multiple assays ($n = 3$). **$p < 0.01$; Student's *t*-test, when compared with the control.

rats. We repeated similar experiments twice, and obtained replicated results (Exp. 1 and 2). These results indicate that the susceptibility of the rat anterior pituitary cells to PrRP is enhanced in lactating rats.

We examined the expression levels of PrRP receptor (i.e., UHR-1) mRNA in the reproductive process by means of quantitative RT-PCR as described previously *(9)*. As

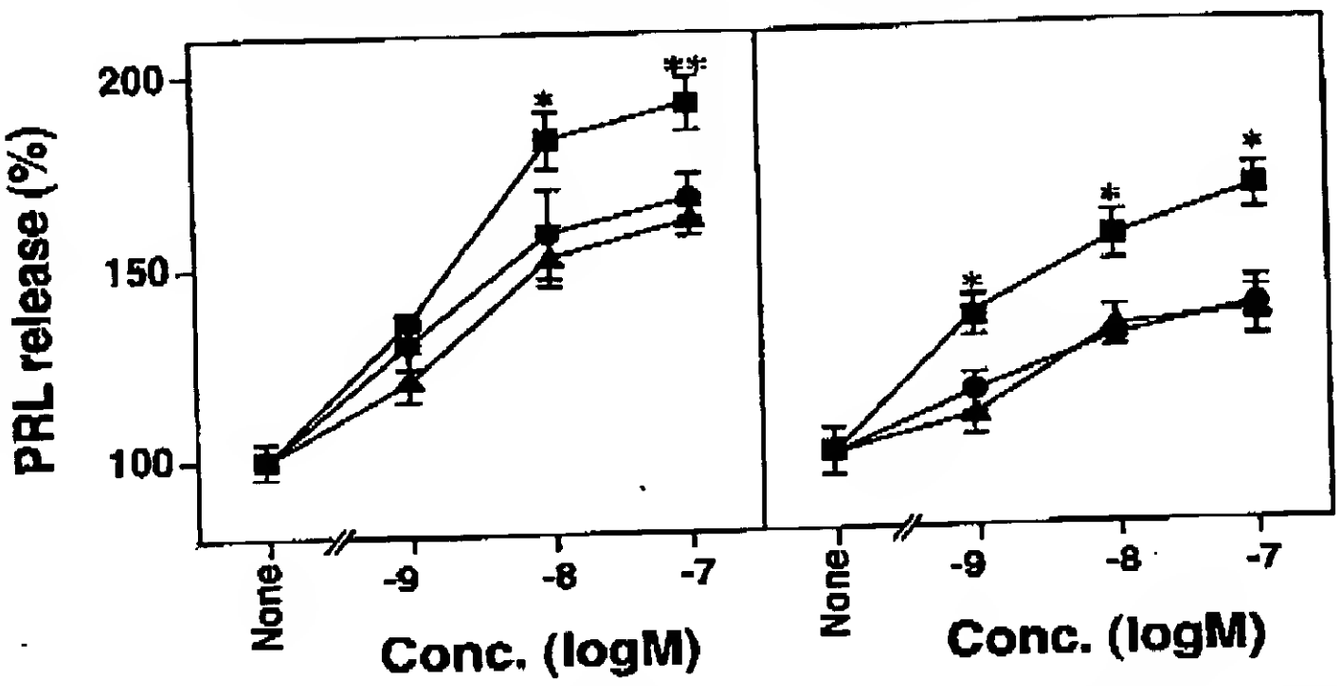

Fig. 6. Responses to PrRP in anterior pituitary cells prepared from female rats at different reproductive stages. Anterior pituitary cells were prepared from female rats of random cycle (▲), 19 d in pregnancy (●), or 11 d in lactation (■), respectively. Culture supernatants were collected 1 h after adding the indicated concentrations of rat PrRP31. Experiment 1 (**left**): PRL concentrations of the control without the sample were 439, 359, and 317 ng/mL in random-cycle, pregnant, and lactating rats, respectively. Data are expressed as mean values ± SEM of percentages in PRL release with each sample in comparison with the control in multiple assays ($n = 6$), except for the controls ($n = 12$). Experiment 2 (**right**): PRL concentrations of the control without the sample were 492, 409, and 412 ng/mL in random-cycle, pregnant, and lactating rats, respectively. Data are expressed as mean values ± SEM of percentages in PRL release with each sample in comparison with the control in multiple assays ($n = 3$), except for the controls ($n = 6$). * $p < 0.05$; ** $p < 0.01$; Student's t-test, when compared to the release in cells from random-cycle rats.

shown in Fig. 7A, the expression level of PrRP receptor mRNA increased in the pituitary in pregnancy and lactation. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA, which we examined as an internal control, also increased in the same periods (Fig. 7B). In pregnancy, the increase in PrRP mRNA appeared to be greater than that of G3PDH, but the ratio between the two mRNAs in lactating rats returned to the comparable level of that in random-cycle rats. On the other hand, the yield of cells obtained from the pituitary did not increase as drastically as PrRP and G3PDH mRNAs did (i.e., 4.8×10^6, 5.1×10^6, and 5.5×10^6 cells/pituitary in random-cycle, pregnant, and lactating female rats, respectively). However, that of poly(A)$^+$ RNA apparently increased during pregnancy and lactation (i.e., 0.43, 0.79, and 1.48 μg/pituitary in random-cycled, pregnant, and lactating rats, respectively), suggesting that the content of PrRP or G3PDH mRNA increases in each cell level. These results indicate that PrRP receptors in the pituitary change considerably in cellular levels at each reproductive stage.

It is well known that treatment with estrogen in vivo enhances the plasma PRL level, accompanied by an increase in the number of lactotrophs *(1,14–16)*. We therefore examined the effect of treatment with estrogen on the response of the anterior pituitary cells to PrRP. We administered β-estradiol 3-benzoate to male rats 3 d before the assay and examined the effect of PrRP on the PRL release after a 4-d culture of the cells (Fig. 8). PrRP slightly promoted PRL release (i.e., about 120–130% compared with the control) from the anterior pituitary cells prepared from untreated male rats. On the other hand, the PRL release of the rat anterior pituitary cells prepared from the estrogen-treated rats was enhanced to about 150–175% by PrRP, indicating that the estrogen treatment increased the susceptibility of the anterior pituitary cells to PrRP.

Discussion

Because culture conditions considerably influenced the responses of the primary-cultured rat anterior pituitary cells to PrRP, we determined the proper conditions under which PrRP could exhibit an ample PRL release–promoting activity. Under the conditions we employed here, PrRP showed comparable activity to TRH in the promotion of PRL release in in vitro culture. We could detect the promotion of PRL release by PrRP using anterior pituitary cells prepared from normal male and random-cycle, pregnant, and lactating female rats, but those prepared from lactating rats were the most sensitive to PrRP.

On the other hand, Samson et al. *(10)* have reported that PrRP was less potent in PRL release–promoting activity than TRH under their culture conditions. We speculate that their culture conditions might result in reduced responsiveness of the rat anterior pituitary cells to PrRP. Several studies indicate that the procedure to disperse anterior pituitary cells sometimes considerably influences their functions in culture *(11,17–19)*. Although Samson et al. *(10)* have employed trypsin to prepare a cell suspension of rat anterior pituitary cells, Nakano et al. *(19)* have reported that the dispersion of the rat anterior pituitary cells with trypsin sometimes seriously affects the cell's hormone secretions including PRL and concluded that collagenase

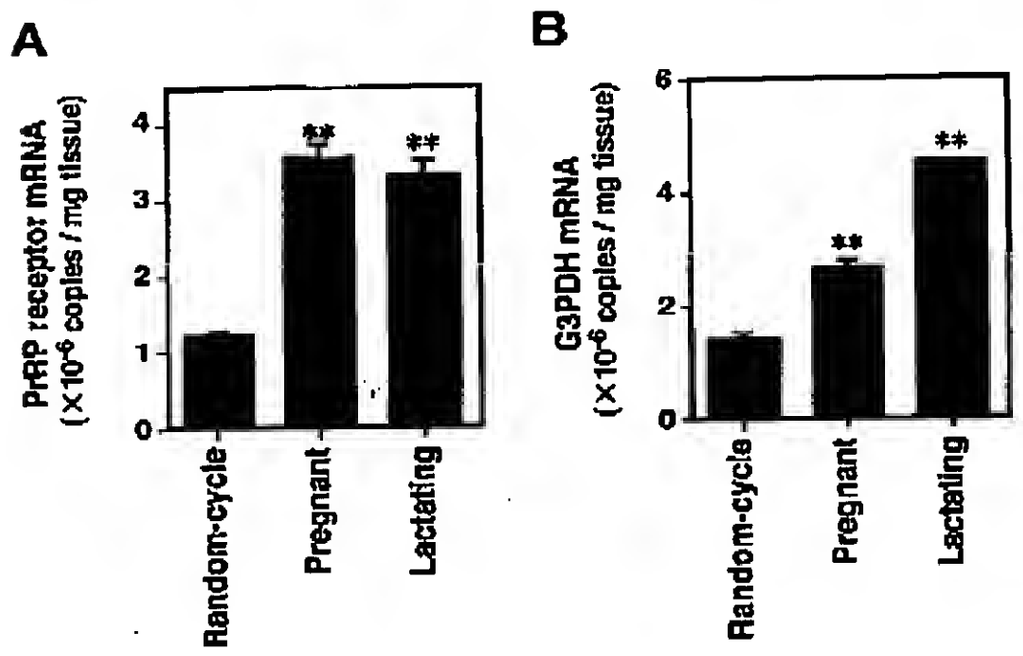

Fig. 7. Quantitative analyses for the expression levels of PrRP receptor mRNA in rat pituitaries in reproductive stages. (A) Quantification of PrRP receptor mRNA; (B) quantification of G3PDH mRNA. Pituitaries from random-cycle, pregnant, and lactating female rats were pooled, respectively, and then poly(A)$^+$ RNA prepared from them was subjected to the quantification of PrRP receptor (UHR-1) or G3PDH (internal control) mRNA expression levels as described previously *(9)*. Data are expressed as mean values ± SEM of copy number per milligram of tissue in quadruplicate analyses. ** $p < 0.01$; Student's *t*-test, when compared to the expression level in random-cycle rats.

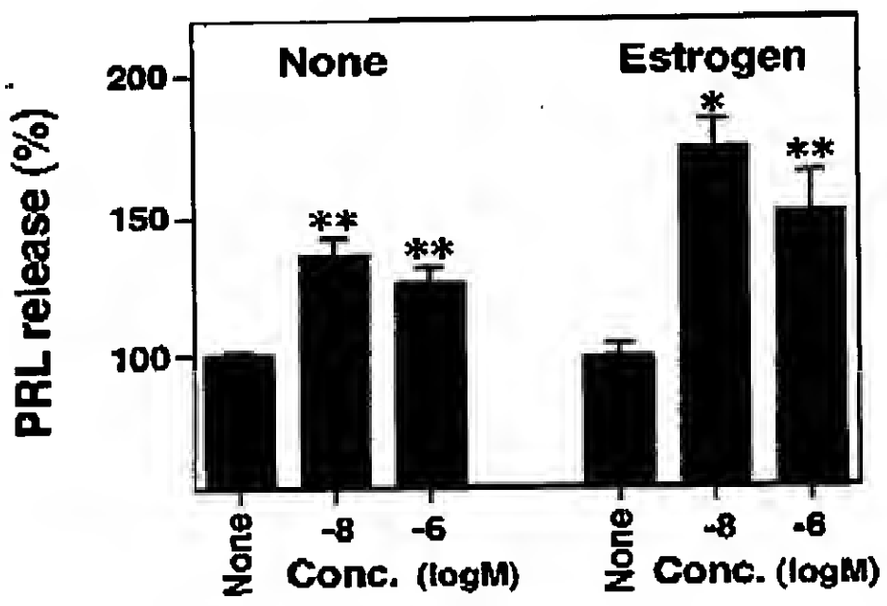

Fig. 8. Responses to PrRP in anterior pituitary cells prepared from male rats treated with estrogen. Anterior pituitary cells were prepared from male rats with or without administering β-estradiol 3-benzoate 3 d before the assay. They were cultured for 4 d, and then culture supernatants were collected 1 h after adding bovine PrRP31 at the indicated concentrations. PRL concentrations of the control without the sample were 166 and 187 ng/mL in untreated and estrogen-treated rats, respectively. Data are expressed as mean values ± SEM of percentages in PRL release with each sample in comparison with the control in multiple assays ($n = 4$). * $p < 0.05$; ** $p < 0.01$; Student's *t*-test, when compared with the control.

treatment followed by a 4-d culture would be suitable to analyze PRL secretion. Shiota et al. *(12,13)* have established the procedure to mildly disperse the anterior pituitary cells using collagenase and pancreatin, as a minor modification of the method reported by Vale et al. *(11)*.

On the basis of thes studies, we used a combination of collagenase and pancreatin, which was thought to be milder for the cells in the dispersion. It was intriguing that culture term considerably influenced the susceptibility of the rat anterior pituitary cells to PrRP. Although the short-term culture for 1 d was enough to detect PRL release induced by TRH, the longer culture term for 4 d was required to obtain a better response to PrRP. These results indicate that rat anterior pituitary cells responsive to PrRP or PrRP receptor itself may be more sensitive to certain enzymatic treatments than those to TRH or TRH receptor, and adequate culture conditions are essential to provide the cells for better responses to PrRP.

In the reproductive stages, plasma PRL levels begin to increase after parturition and are high during lactation in rats. The susceptibility of the anterior pituitary cells to TRH in PRL secretion is reportedly enhanced by suckling stimuli *(20,21)*. In our study, the anterior pituitary cells prepared from lactating rats were more sensitive to PrRP in PRL secretion than those from random-cycle and pregnant rats. We found that the expression of PrRP receptor mRNA in the pituitary rose during pregnancy and lactation. Because lactotrophs increase in the pituitary during pregnancy and lactation, the increase in PrRP receptor mRNA appears to parallel that of lactotrophs. However, note that the cells prepared from pregnant rats were not as susceptible to PrRP as those from lactating rats. Therefore, the expression level of PrRP receptor mRNA does not seem to regulate solely the susceptibility of the anterior pituitary cells to PrRP. Binding experiments using a proper labeled ligand would give more information about the expression level of PrRP receptors. Further studies will be required to reveal the mechanism responsible for the enhanced susceptibility of the anterior pituitary cells obtained from lactating rats. In addition, we found that the expression level of PrRP mRNA increased in the medulla oblongata during pregnancy (unpublished data). The expression levels of both PrRP and its receptor changed during pregnancy and lactation, suggesting that they play a certain role in the reproductive process.

Although PrRP significantly promoted PRL release from the anterior pituitary cells prepared from both male and nonlactating female rats, its effect was marginal in these cases. However, the in vivo administration of estrogen afforded better responses to PrRP in male rats. We have observed that the in vivo administration of PrRP caused a specific increase in PRL in plasma in rats, although higher doses of PrRP were required to increase PRL levels in comparison with TRH *(22)*. The in vivo effect of PrRP on PRL release was considerably changed by the estrous cycle and sex in rats; that is, female rats especially in proestrus and estrus were more sensitive to PrRP than male rats *(22)*. Recently, it was reported that treatment with estrogen obviously enhanced the effect of PrRP on the elevation of

plasma PRL levels in vivo *(23)*. These facts suggest that the susceptibility of the rat anterior pituitary cells to PrRP is regulated by physiological mechanisms including estrogen. Alternatively, PrRP would work as a potent PRL-releasing factor under certain physiological conditions.

In comparison between PrRP and TRH, tissue distribution of their mRNA was quite different in the rat brain; that is, the highest expression of PrRP mRNA was observed in the medulla oblongata, whereas that of TRH mRNA was in the hypothalamus *(9)*. It has also been noted that the distribution of immunoreactive PrRP is distinct from that of the known hypothalamic hormones in the rat brain *(24,25)*. In the immunohistochemical analyses, we could not detect an apparent projection of PrRP-positive neurons to the median eminence, where the known hypothalamic hormone neurons are projected in order to act on the pituitary via the portal vessels *(26,27)*. We therefore consider that the mechanism for PrRP acting on the pituitary would differ from those for the known hypothalamic hormones. For example, Matsumoto et al. *(24)* have reported that PrRP is detected in the adrenal or posterior pituitary at relatively high levels in rats. PrRP present in these organs might be delivered to the anterior pituitary. Iijima et al. *(26)* have proposed the possibility that PrRP produced in the brain might be delivered into the systemic circulation and the cerebrospinal fluid because PrRP-positive neurons are projected to ependymal cells adjacent to the ventricle or capillary blood vessels.

Our recent studies suggest that PrRP takes part not only in the regulation of various hormone secretions including oxytocin as well as PRL *(27)*, but also in neuronal transmission or modulation *(26)*. We believe that further studies on PrRP and its receptor will provide clues that will help reveal novel mechanisms for the regulation of the endocrine and central nervous systems.

Materials and Methods

Animals

Adult male (10 wk old) or female (12–14 wk old) Fischer 344/N rats (SLC Japan, Shizuoka, Japan) were housed under controlled temperature and lighting conditions (lights on from 8:00AM to 8:00 PM) and were supplied with water and food ad libitum. All experiments using rats were conducted according to a guideline of the internal animal care and use committee. Pregnant and lactating female rats were used 19 d in pregnancy and 10–12 d postpartum, respectively. To examine the effects of estrogen, 50 µg of β-estradiol 3-benzoate (Wako, Osaka, Japan) was subcutaneously injected into the dorsal region of the neck of male rats, and the rats were housed for 3 days before use.

Culture of Anterior Pituitary Cells

Anterior pituitary cells were prepared according to the method reported by Vale et al. *(11)* and Shiota et al. *(12,13)* with minor modifications After the rats were decapitated, the anterior pituitaries were quickly removed and placed in an ice-cold buffer A consisting of 137 m*M* NaCl (Wako), 5 m*M* KCl (Wako), 0.7 m*M* Na_2HPO_4 (Wako), 25 m*M* HEPES (Dojindo, Kumamoto, Japan) at pH 7.3, 50 µg/mL of gentamicin (Gibco-BRL, Grand Island, NY), 50 U/mL of penicillin (Banyu Pharmaceutical, Tokyo, Japan), and 50 µg/mL of streptomycin (Meiji Seika Kaisha, Tokyo, Japan). They were quartered with scissors and then washed with the same buffer. The resultant dissected tissues were incubated at 37°C for 1–1.5 h in a buffer A containing 0.4% collagenase (Boehringer Mannheim, Mannheim, Germany), 10 µg/mL of DNase (Sigma, St. Louis, MO), 0.4% bovine serum albumin (BSA) (Sigma), and 0.2% glucose (Wako). The pituitary cells were dispersed by gentle trituration using a plastic pipet, and centrifuged at 320*g* for 10 min. The cell pellet was subsequently suspended in a buffer A containing 0.25% pancreatin (Sigma), and then the suspension was incubated at 37°C for 8 min. After adding fetal bovine serum (FBS) (BioWhittaker, Walkersville, MD) to stop the enzymatic digestion, the suspension was centrifuged at 220*g* for 6 min.

The cell pellets were suspended in Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical, Tokyo, Japan) containing 3.7 g/L of $NaHCO_3$, 10% FBS, 20 m*M* HEPES at pH 7.3, 50 U/mL penicillin, and 50 µg/mL of streptomycin (DMEM-I), and then filtrated through a nylon mesh (cell strainer; Becton Dickinson, Bedford, MA) to remove undigested tissue blocks. A typical yield was 1.5–5.5 × 10^6 cells/pituitary in female rats and 1.4–2.0 × 10^6 cells/pituitary in male rats. Viability of the cells determined with a trypan blue solution (Gibco-BRL) was usually more than 90%. After being washed twice with DMEM-I, the cells were cultured at 1.5 × 10^5 cells in analyses for PRL and GH secretions or at 5.0 × 10^5 cells in those for TSH, LH, FSH, and ACTH secretions in 1 mL of DMEM-I in poly-D-lysine-coated 24-well plates (Becton Dickinson) at 37°C under humidified atmosphere of 5% CO_2 in air. The culture medium in the wells was replaced every 3 d with fresh medium.

Synthetic Peptides

Bovine, human, and rat PrRP31 or PrRP20 were synthesized as described previously *(7,28)*. TRH, human GHRH and LHRH, and ovine CRH were purchased from Peptide Institute (Osaka, Japan).

Analyses for Release of Pituitary Hormones Induced by Peptides

After the anterior pituitary cells were cultured for 1 d or 4 d, they were washed three times with DMEM-II (DMEM containing 0.2% BSA and 20 m*M* HEPES at pH 7.3), and then incubated in 1 mL of the same medium at 37°C for 15 min to 1 h. After being washed twice, the cells were incubated with a sample dissolved in 1 mL of the medium. After incubation for 1 h or the time desired, culture

supernatants were harvested, centrifuged, and then stored at –30°C until used. Concentrations of PRL, GH, TSH, FSH, and LH were determined using Biotrak RIA assay systems (Amersham, Buckinghamshire, England). The concentration of ACTH was determined using a DPC·ACTH kit (Diagnostic Products, LA).

Quantitative Analyses for Rat PrRP Receptor mRNA by RT-PCR

The expression levels of UHR-1 mRNA were determined as described previously *(9)*. We prepared poly(A)$^+$ RNAs from pituitaries of random-cycle, pregnant, and lactating female rats by Isogen (Wako) and Micro-FastTrack (Invitrogen, San Diego, CA). We subsequently synthesized cDNA from each poly(A)$^+$ RNA (160 ng) treated with deoxyribonuclease I (amplification grade; Gibco-BRL) in the presence of 2.5 μ*M* random hexanucleotides (Takara Shuzo, Kyoto, Japan) and 10 U of AMV reverse transcriptase XL (Life Sciences, Petersburg, FL) at 42°C for 30 min. The primer pairs 5'-CCTGCTGGCCATTCTCCTGTCTTAC-3' and 5'-GGGTCCAGGTCCCGCAGAAGGTTGA-3' were used for the amplification to yield a 204-bp length of the PCR products. Primers to detect rat G3PDH were purchased from Clontech (Palo Alto, CA). PCR was carried out in a 25-μL reaction mixture containing 4 μL of diluted cDNA solution, 0.5 m*M* dNTPs, 5 pmol of each primer, and 0.5 μL of KlenTaq polymerase mix (Clontech). To amplify the UHR-1 cDNA, PCR was performed for 26 cycles at 98°C for 10 s and 68°C for 25 s. A 5-μL aliquot of each PCR product was electrophoresed on a 4% agarose gel and then stained with ethidium bromide. After gel images had been captured by a FOTO/Ecripse (Fotodyne, Hartland, WI) with a charge-coupled device camera, the intensity of the ethidium bromide luminescence was analyzed by a densitometry program (1-D Basic; Advanced American Biotechnology, Fullerton, CA). To obtain a calibration curve, we measured the known amounts of plasmid containing UHR-1 on the basis of optical density and then subjected it to PCR. The resultant PCR products were analyzed in the same manner as just above. No band was detected after PCR in the samples that had not been subjected to reverse transcription.

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A prolactin-releasing peptide in the brain

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A prolactin-releasing peptide in the brain

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Hypothalamic peptide hormones regulate the secretion of most of the anterior pituitary hormones, that is, growth hormone, follicle-stimulating hormone, luteinizing hormone, thyroid-stimulating hormone and adrenocorticotropin[1,2]. These peptides do not regulate the secretion of prolactin[1,2], at least in a specific manner, however. The peptides act through specific receptors, which are referred to as seven-transmembrane-domain receptors or G-protein-coupled receptors[3–7]. Although prolactin is important in pregnancy and lactation in mammals, and is involved in the development of the mammary glands and the promotion of milk synthesis[8,9], a specific prolactin-releasing hormone has remained unknown. Here we identify a potent candidate for such a hormone. We first proposed that there may still be unknown peptide hormone factors that control pituitary function through seven-transmembrane-domain receptors. We isolated the complementary DNA encoding an 'orphan' receptor (that is, one for which the ligand is unknown). This receptor, hGR3, is specifically expressed in the human pituitary. We then searched for the hGR3 ligand in the hypothalamus and identified a new peptide, which shares no sequence similarity with known peptides and proteins, as an endogenous ligand. We show that this ligand is a potent prolactin-releasing factor for rat anterior pituitary cells; we have therefore named this peptide prolactin-releasing peptide.

We searched seven-transmembrane-domain receptors (7TMRs) with a polymerase chain reaction (PCR) method, and isolated from the human pituitary an orphan 7TMR, hGR3, that is nearly identical to GPR10 (ref. 10) and a human counterpart of rat UHR-1 (ref. 11). Quantitative analyses of UHR-1 messenger RNA by reverse transcription PCR (RT-PCR) revealed that, of over 40 different tissues, the pituitary expressed UHR-1 mRNA at the highest level, whereas the brain, spinal cord, adrenal gland, and femur expressed UHR-1 mRNA at moderate levels. *In situ* hybridization analysis indicated that the anterior lobe abundantly expressed UHR-1 mRNA, suggesting that UHR-1 or hGR3 is particularly important in the regulation of anterior pituitary function.

On the basis of specific signal transduction in CHO cells expressing hGR3 (CHO-19P2) compared with in CHO cells transfected with a vector plasmid lacking hGR3 cDNA, we searched for an endogenous ligand of hGR3 in tissue extracts. We used several different assays for detecting signal transduction, and detected a specific response of CHO-19P2 cells to bovine hypothalamic extract when we used the arachidonic acid metabolite release assay. We used this assay as the basis for the purification of an hGR3 ligand through a combination of chromatographic procedures. The arachidonic acid metabolite-releasing activities separated into three peaks (P1, P2 and P3) with Vydac C_{18} column chromatography. As the activity of P1 seemed to be less than those of P3 and P2, we purified P3 and P2 by Vydac diphenyl column and μRPC C_2/C_{18} column chromatography. As shown in Fig. 1a, two peaks of activity further separated from P3 by μRPC C_2/C_{18} column chromatography. Both peaks derived from P3 gave the same partial amino-terminal amino-acid sequence SRAHQHXMEIRTPDINPAXYAGRGIRPVG (X, unidentified residue), indicating that the difference between the two peaks might be due to a minor modification of the same peptide, such as oxidation of the methionine residue. In the case of P2, the activity was detected as a single peak (Fig. 1b). The purified P2 gave a partial N-terminal amino-acid sequence, which partly overlapped with that of P3, of TPDINPAWYAGRGIRPVGR.

We isolated bovine, rat and human cDNAs encoding the P2 and P3 peptide sequences from the brain of each species, using the purified peptide sequences as a basis for isolation of the cDNAs (Fig. 2). Although the bovine cDNA encoded a protein of 98 amino-acid residues, its N-terminal portion before Ser 23 showed the

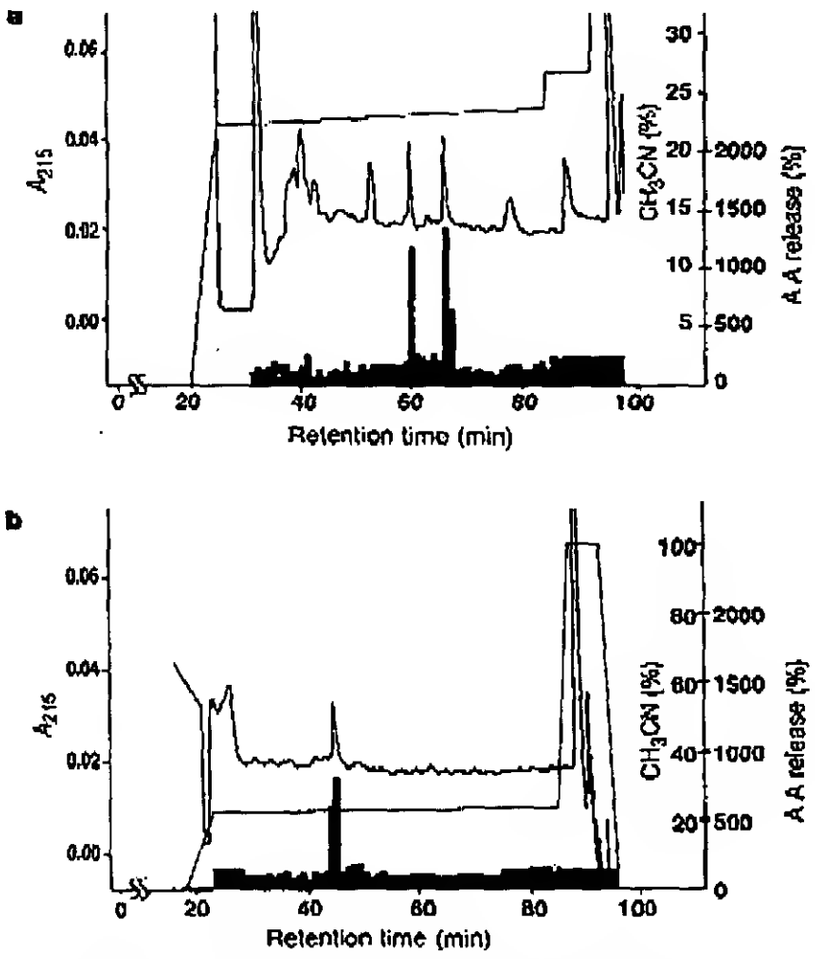

Figure 1 *Purification of peptide ligands for hGR3 from bovine hypothalamic extract.* The arachidonic acid metabolite (AA)-releasing activity (black areas) of each fraction is expressed as a percentage of the amount of [^{3}H]AA released from control CHO-19P2 cells in a single assay. Thick jagged trace, A_{215}; thin line, percentage of CH_3CN. **a**, *The profile of peptide P2 in μRPC C_2/C_{18} column* chromatography using a SMART system. Elution was performed with a linear gradient of 22.0–23.5% CH_3CN. **b**, The profile of peptide P2 in μRPC C_2/C_{18} column chromatography with a linear gradient of 21.5–23.0% CH_3CN.

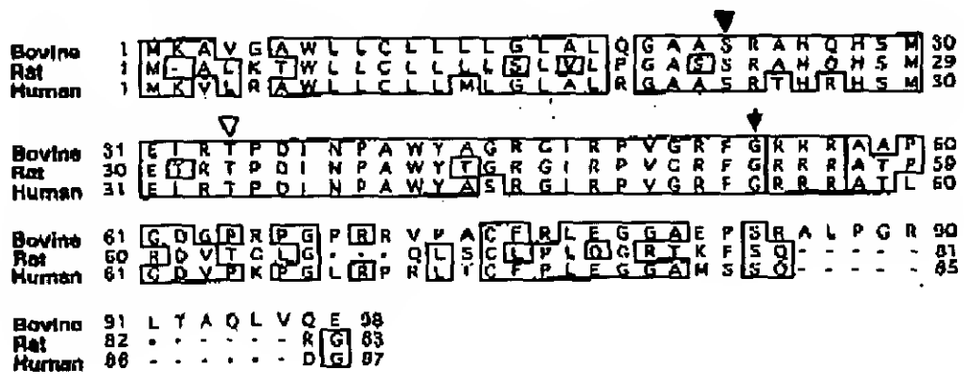

Figure 2 Amino-acid sequences of bovine, rat and human preproproteins containing PrRP. These sequences were deduced from cDNAs. The filled and open arrowheads indicate the N termini of PrRP31 and PrRP20, respectively. The arrow indicates a glycine residue that is presumed to react as an amide donor. The triplets of basic amino-acid residues that constitute the typical motif of a protcolytic cleavage site are boxed with a thick line. Amino-acid residues with identical sequence in at least two of the species are boxed with a thin line.

typical profile of a secretory signal peptide[12], indicating that P3 was generated through cleavage of the signal sequence. The P2 peptide sequence, which starts from Thr 34, indicates that the P2 peptide may be a truncated form of the P3 peptide. A typical proteolytic cleavage motif, comprising the basic amino-acid repeat Arg 55, Arg 56, Arg 57, was conserved among the species, as was Gly 54. This suggests that after cleavage between Gly 54 and Arg 55, Phe 53 at the carboxy terminus of the predicted mature peptides might be amidated by reacting with Gly 54 as an amide donor.

These results indicate that the preproprotein encoded by the bovine cDNA may generate at least two forms of mature peptide as naturally occurring endogenous ligands, that is, SRAHQHSM EIRTPDINPAWYAGRGIRPVGRF-NH_2 and TPDINPAWYAGRGIR PVGRF-NH_2, thought to correspond to the purified P3 and P2, respectively. We have named these peptides prolactin (PRL)-releasing peptides (PrRPs). PrRP31 and PrRP20 correspond to peaks P3 and P2, respectively. These putative mature peptide sequences were highly conserved among the species. We analysed the tissue distribution of PrRP mRNA in rats by the quantitative RT-PCR method, and found that the medulla oblongata expressed the highest level of PrRP mRNA, whereas the hypothalamus expressed PrRP mRNA at a moderate level. However, when determined on the basis of the arachindonic acid metabolite release assay, the content of bioactive PrRP in tissue extracts was highest in the hypothalamus.

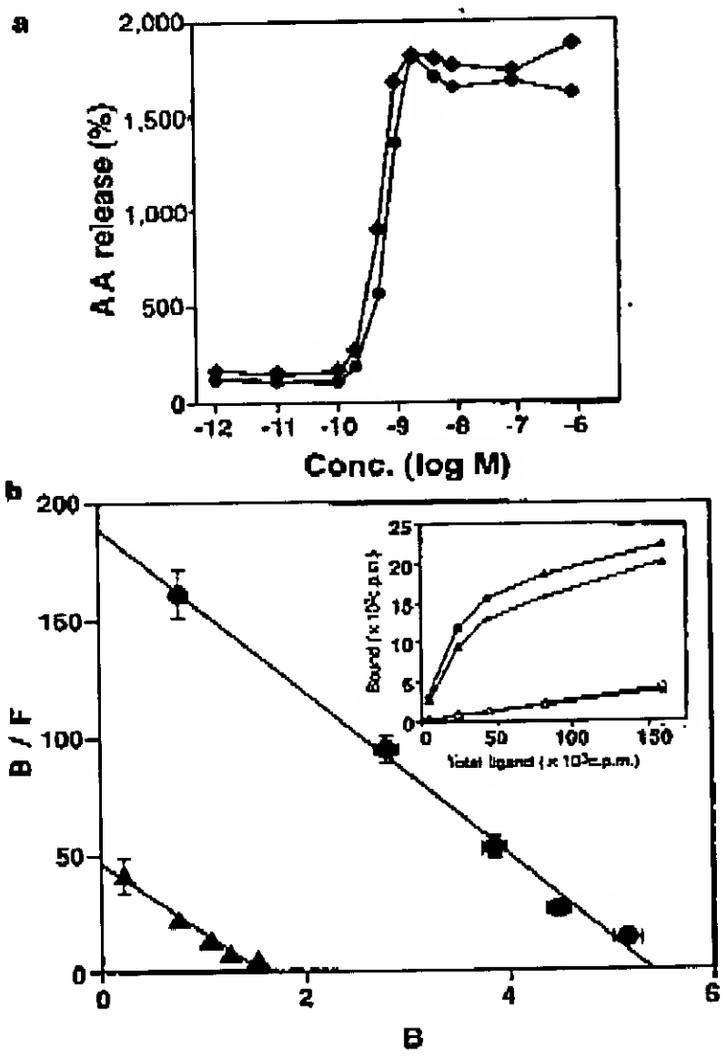

Figure 3 Specific interaction of synthetic PrRPs with hGR3 and UHR-1. a, Specific arachidonic acid metabolite (AA) release from CHO-19P2 cells is induced by PrRPs. CHO-19P2 cells were incubated with the indicated concentrations of bovine PrRP31 (circles) or PrRP20 (diamonds), respectively. Values represent the means of percentages of [^{3}H]AA released in relation to a control in duplicate assays. The amount of [^{3}H]AA released from the control was 867 c.p.m. **b**, Scatchard analysis of the binding of bovine PrRP31 to hGR3 and UHR-1. Scatchard plots of binding of ^{125}I-labelled bovine PrRP31 to CHO-19P2 (circles) and CHO-UHR-1 (triangles) cells are shown. B, bound (p mol mg^{-1} protein); F, free (nM). The inset panel represents the saturation binding data: symbols indicate the total binding of ^{125}I-labelled bovine PrRP31; in the presence of 1 μM unlabelled bovine PrRP31, to the membrane fraction of CHO-19P2 cells (filled circles) and CHO-UHR-1 cells (filled triangles), and nonspecific binding to CHO-19P2 cells (open circles) and CHO-UHR-1 cells (open triangles).

We synthesized bovine, rat and human PrRP31 and PrRP20, and examined their ability to induce arachidonic acid metabolite release from CHO-19P2 cells and CHO cells expressing UHR-1 (CHO-UHR-1 cells). Synthetic PrRP31 and PrRP20 both promoted arachidonic acid metabolite release by CHO-19P2 (Fig. 3a) and CHO-UHR-1 cells, but not by mock-transfected CHO cells. We next examined the binding of synthetic PrRPs to hGR3 and UHR-1. ^{125}I-labelled bovine PrRP31 specifically bound to membrane fractions prepared from both CHO-19P2 and CHO-UHR-1 cells. Competitive binding experiments showed that unlabelled PrRPs inhibited the binding of ^{125}I-labelled bovine PrRP31 to both CHO-19P2 and CHO-UHR-1 cells, in a dose-dependent manner. As shown in Fig. 3b, Scatchard analysis showed that both CHO-19P2 and CHO-UHR-1 cells expressed a single class of high-affinity binding site for PrRP31. The dissociation constants (K_d) were 2.6×10^{-11} M and 2.5×10^{-11} M, and the maximal binding sites (B_{max}) were 4.8 and 1.3 pmol mg^{-1} protein, in CHO-19P2 and CHO-UHR-1 cells, respectively. However, PrRP31 with a non-amidated carboxyl group C terminus exhibited drastically decreased arachidonic acid metabolite-releasing and receptor-binding activities towards CHO-19P2 cells, indicating that an amidated C terminus is needed for PrRPs to interact with the receptor.

To determine the physiological effects of PrRP, we first studied its effect on a rat pituitary adenoma derived cell line, RC-4B/C[13], in which we had found the apparent expression of UHR-1 mRNA. Of the anterior pituitary hormones, only PRL was constitutively secreted at a detectable level by RC-4B/C cells under our culture conditions. Addition of PrRP31 to the culture increased the secretion of PRL from RC-4B/C cells within 30 min (Fig. 4a). We therefore expected that PrRP might act on lactotrophs to promote PRL secretion. We prepared anterior pituitary cells from lactating female rats, as the numbers of PRL-producing cells, that is, the lactotrophs, are increased in such rats[9]. We tested the effect of PrRP on hormone secretion from these cells. PrRP31 promoted PRL secretion within 15 min to 1 h of application (Fig. 4b). Thyrotropin-releasing hormone (TRH) is a potent factor that is known to be capable of promoting PRL secretion[8,9]; PrRP31 was comparable to TRH in its potency. However, PrRP31 did not influence the secretion of the other anterior pituitary hormones, that is, growth hormone, follicle-stimulating hormone, luteinizing hormone, thyroid-stimulating hormone or adrenocorticotropin. In addition, PrRP31 immediately promoted the secretion of PRL from rat anterior pituitary cells in a perifusion assay (Fig. 4c), indicating that PrRP may stimulate lactotrophs directly to secrete PRL. Although some other known peptides, such as vasoactive intestinal polypeptide, oxytocin, substance P, neurotensin, arginine–vasopressin, pituitary adenylate cyclase-activating polypeptide and galanin, have been reported to show PRL-releasing activity *in vitro* or *in vivo*[14–19], these peptides did not show apparent PRL-releasing activity in primary cultured rat anterior pituitary cells, at least under our experimental conditions (Fig. 4d).

Arachidonic acid metabolism is important as a signal-transduction pathway in PRL secretion by pituitary cells[20], although we found that PrRP could induce Ca^{2+} influx and a partial suppression of cyclic AMP production as well as arachidonic acid metabolite release in CHO-19P2 cells. As shown in Fig. 5a, PrRP induced pronounced arachidonic acid metabolite release as well as PRL secretion even in primary cultured rat anterior pituitary cells. In addition, as shown in Fig. 5b, the specific phospholipase A_2 inhibitor BPB (4-bromophenacylbromide) and the lipoxygenase inhibitor NDGA (nordihydroguaiaretic acid) attenuated both basal and PrRP-induced PRL secretion; however, the cycloxygenase inhibitor indomethacin had no such effect. These results show that arachidonic acid metabolism is closely linked to PrRP-induced PRL secretion, and that the lipoxygenase pathway is at least partly responsible for this effect.

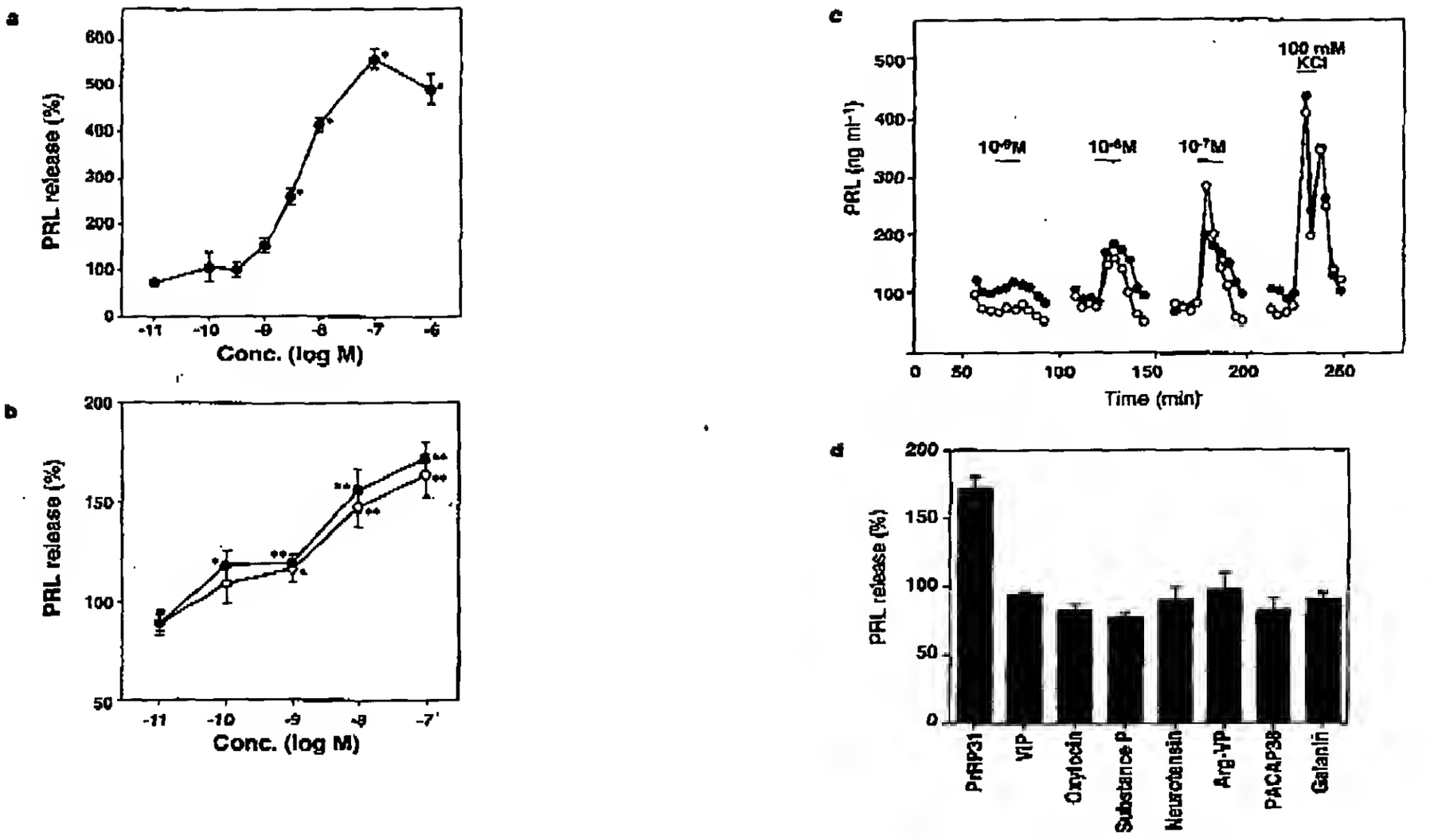

Figure 4 *Promotion of PRL secretion from rat anterior pituitary cells by PrRP.* **a**, Promotion of PRL secretion from RC-4B/C cells by bovine PrRP31. PRL concentrations represent the means of percentages ± s.e.m. (vertical bars) relative to a control (100%) without PrRP31, from quadruplicate assays. PRL released by the control was 1.2 ng ml^{-1}. **b**, Promotion of PRL secretion from primary cultured rat anterior pituitary cells in static incubation by bovine PrRP31. The indicated concentrations of bovine PrRP31 (filled circles) or TRH (open circles) were added to the culture. PRL concentrations are shown as for **a**. PRL released from the control was 518 ng ml^{-1}. **c**, Promotion of PRL secretion from rat anterior pituitary cells by rat PrRP31 in a perifusion assay. Rat PrRP31 (filled circles) and TRH (open circles) at indicated doses were added to the system in different chambers at the intervals indicated by the horizontal bars. PRL concentrations taken every 3 min are indicated. **d**, Comparison of the ability of PrRP and of known peptides to promote PRL secration in static incubation. Bovine PrRP31 and the other peptides indicated were used at 10^{-7} M. VIP, vasoactive intestinal polypeptide; Arg-VP, arginina–vasopressin; PACAP38, pituitary adenylate cyclase-activating polypeptide. Single asterisks indicate $P < 0.05$; double asterisks indicate $P < 0.01$ (when compared with control); Student's *t*-test.

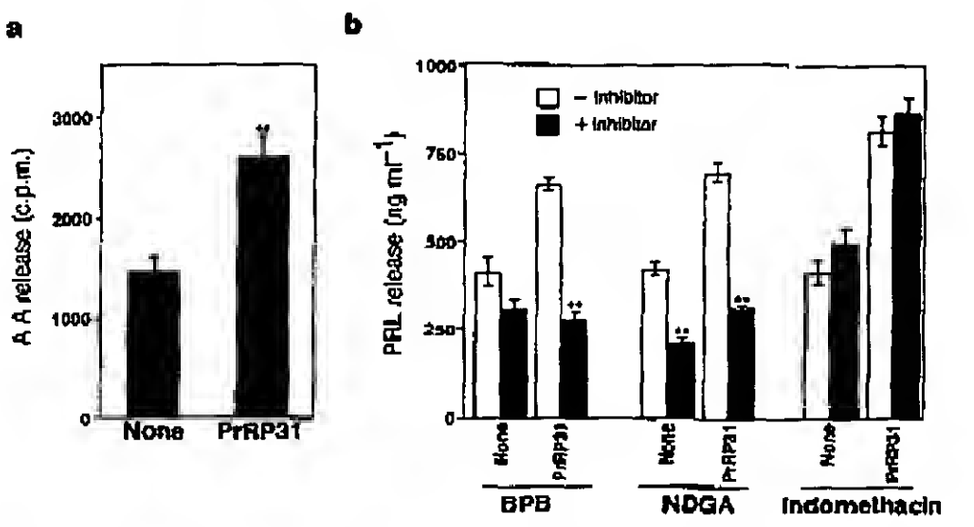

Figure 5 Relation between arachidonic acid metabolism and PRL secretion induced by PrRP in primary cultured rat anterior pituitary cells. Values represent the means of quadruplicate assays ± s.e.m. (vertical bars). Double asterisks indicate $P < 0.01$ (when compared with the control); Student's *t*-test. **a**, Release of arachidonic acid metabolites (AA) induced by rat PrRP31. AA release in the presence or absence (none) of rat PrRP31 at 10^{-7} M is shown. **b**, Effect of inhibitors on the secretion of PRL induced by rat PrRP31. Anterior pituitary cells untreated or treated with rat PrRP31 at 10^{-8} M were incubated in the presence (filled columns) or absence (white columns, controls) of each inhibitor at 60 μM.

The 7TMR gene family comprises many receptors which control various physiological functions. Many orphan 7TMRs have been discovered with the recent development of genome and cDNA research. However, only a few new peptide ligands have been identified for such orphan 7TMRs. Such peptides include orphanin FQ/nociceptin[21,22], a snail neuropeptide[23] and the orexins[24]. The strategy we used here would be applicable to the identification of many other unknown factors that regulate certain functions of various organs through orphan 7TMRs. The known hypothalamic hormones that regulate secretion of anterior pituitary hormones act on the pituitary through the hypophyseal portal vessel[1,2]. As levels of biologically active PrRP were highest in the hypothalamus, PrRP might also act on the anterior pituitary through the hypothalamus/portal vessel/pituitary axis, although this is uncertain at present. Further studies will be needed to reveal the physiological significance of PrRP and its receptor *in vivo*, but we observed that the levels of expression of the mRNA for PrRP and its receptor apparently fluctuate in the medulla oblongata and pituitary during pregnancy and lactation, respectively, in rats, indicating that levels of PrRP and its receptor are closely related to the regulation of certain reproductive processes. PRL secretion is regulated in a complicated manner *in vivo* in various physiological situations[8,9]. We assume that PrRP is important in the regulation of PRL secretion, especially in reproductive processes. PrRP and its receptor might also have functions in other tissues, including the central nervous system. Further studies of PrRP and its receptor will give us new insights into the regulatory mechanism of pituitary function and other physiological phenomena. □

Methods

Arachidonic acid metabolite release assay. We established CHO-19P2 and CHO-UHR-1 cells[3] by transfecting CHO cells lacking the dihydrofolate reductase gene with expression vector plasmids containing hGR3 or UHR-1 cDNAs. After culturing the CHO cells in 24-well plates at 5×10^4 cells per well for 24 h, and rat anterior pituitary cells in 24-well plates at 5×10^5 cells per well for 4 days, we added [^{3}H]arachidonic acid (NEN/Dupont) to each well at 0.25 or 2.0 μCi per well, and incubated the plates for a further 16 h. Then we washed the cells, added a sample, incubated the plates for 15 or 30 min, and measured the amount of [^{3}H]arachidonic acid released into the culture supernatant.

Purification of peptide ligands for hGR3. We boiled bovine hypothalamic tissue (2 kg), homogenized it in 1 M acetic acid, and collected the supernatant. We fractionated the supernatant on a C_{18} open column (PrepC_{18} 125Å:Waters) with stepwise increments of 10%, 30% and 50% CH_3CN in 0.05% trifluoroacetic acid (TPA) in water. We then fractionated the 30% CH_3CN fraction on HiPrep CM–Sepharose FF (Pharmacia), with stepwise increments of 100, 200, 500 and 1,000 mM CH_3COONH_4 (pH 6.4) in 10% CH_3CN. After precipitation with acetone, the 200 mM CH_3COONH_4 fraction was serially fractionated on a Resource RPC (Pharmacia) column with a linear gradient of 15–30% CH_3CN, a Resource S (Pharmacia) column with a linear gradient of 0–0.7 M NaCl in 50 mM 2-morpholinoethanesulphonic acid (pH 5) containing 10% CH_3CN, and a Vydac C_{18} 218TP5415 (Separations) column with a linear gradient of 20–30% CH_3CN. We further fractionated positive fractions (P3 and P2) on a Vydac diphenyl 219TP5415 (Separations) column with linear gradients of 22–25 and 21–24% CH_3CN for P3 and P2, respectively, and then on a μRPC C_2/C_{18} SC 2.1/10 column (Pharmacia) in a SMART system (Pharmacia) for 60 min at a flow rate of 100 μl min^{-1}. We analysed the N-terminal amino-acid sequences of purified peptides with a protein sequencer (model 492, ABI).

Cloning of cDNAs encoding peptide ligands for hGR3. We isolated cDNA encoding the peptide ligands from poly(A)$^+$ RNA of the bovine hypothalamus by using PCR and rapid amplification of cDNA ends (RACE), with a 3′-RACE system (Gibco BRL), a Marathon cDNA amplification kit (Clontech), and degenerate primers designed on the basis of the purified peptide sequences. We isolated a rat cDNA from poly(A)$^+$ RNA prepared from the dorsal region of the medulla oblongata of Wistar rats, nearly according to the strategy used for the cloning of the bovine cDNA, and then isolated a human cDNA from human brain poly(A)$^+$ RNA (Clontech) with primers designed on the basis of conserved sequences found in bovine and rat cDNAs.

Synthesis of peptides. PrRPs were chemically synthesized with an automatic peptide synthesizer (model 430, ABI). They were also prepared as recombinant peptides produced in *Escherichia coli*[25,26]. Synthetic bovine PrRP31 was labelled with ^{125}I by use of [^{125}I] Bolton–Hunter reagent (NEN/Dupont). This PrRP was used for receptor-binding assays[27].

Assay for hormone secretions. RC-4B/C cells were cultured at 1×10^5 cells per well in 12-well tissue culture microplates for 2 days[13]. Anterior pituitary cells prepared from lactating female F344/N rats were cultured at 1.5×10^5 cells per well in poly-D-lysine-coated 24-well plates (Falcon Biocoat 40414) for 4 days[28]. The cells were then incubated with 1 ml of the medium containing each sample for 15 min to 1 h at 37 °C. Perifusion assays were performed at a flow rate of 0.33 ml min^{-1} (ref. 29). The PRL-secreting ability of the cells was confirmed by using KCl. Amounts of pituitary hormones were determined with assay kits (follicle-stimulating hormone, thyroid-stimulating hormone, luteinizing hormone, growth hormone and PRL, Biotrak RIA/Amersham; adrenocorticotropin, Nippon DPC).

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